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Rural–urban focus of canine visceral leishmaniosis in the far western region of Santa Catarina State, Brazil

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ABSTRACT

The aim of this work was to investigate the occurrence of canine visceral leishmaniosis (CVL) in the far western region of Santa Catarina State, bordering Argentina and Parana State, southern Brazil, where in recent years, VL has been recorded in both dogs and humans. Clinical signs, enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT) and polymerase chain reaction (PCR) were used for *Leishmania* investigation. Among the 252 dogs surveyed, 41 were positive by ELISA assay, 43 in IFAT (titer > 40), and 55 by PCR. From the 48 positive for VL by both serological and molecular methods, 19 (39.6%) presented clinical symptoms of leishmaniosis, 35 (72.9%) were from rural areas, and 13 (27.1%) were from urban areas. This pilot study confirms the occurrence of VL among dogs in the far western region of Santa Catarina, southern Brazil, with high risk of CVL outbreaks and presenting a threat to humans.

1. Short report

Visceral leishmaniosis (VL), caused by *Leishmania* (*Leishmania*) *infantum* Nicolle 1908, is a chronic zoonosis in which domestic dogs are a known reservoir (Cortes et al., 2012). Several epidemiological studies have shown the growing spread of the disease worldwide and in Brazil (Solano-Gallego et al., 2001; Moreno and Alvar, 2002; Alvar et al., 2004; Maia-Elkhoury et al., 2008). According to the Brazilian Health Ministry, between 1980 and 2005, a total of 59,129 human cases were recorded and 82.5% of these occurred in the northeastern region. However, the parasite gradually spread to other regions of the country, particularly to the States of Minas Gerais, São Paulo, and Mato Grosso (Maia-Elkhoury et al., 2008). More recently, outbreaks of CVL have been recorded in southern Brazil (Rio Grande do Sul, Santa Catarina, and Parana State), Paraguay, and principally in Argentina (Salomon et al., 2008; Cruz et al., 2010; Deboni et al., 2011; Dias et al., 2013; Steindel et al., 2013). It is important to highlight that hunting with dogs is a common practice in Argentina, which may be a triggering factor for disease outbreak, because the country has recorded outbreaks of CVL near the border of the states of Rio Grande do Sul and Paraná (Salomon et al., 2008; Cruz et al., 2010). Given this context, surveillance of CVL cases is important because canine infection precedes the disease in humans (Marzochi et al., 2009).

Thus, this study aimed to investigate VL occurrence among dogs in the far western region of Santa Catarina

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State, Brazil. The study was conducted in the municipalities of São Miguel do Oeste and Descanso, latitude 26°43′31″ and longitude 53°31′05″. The climate is humid subtropical (*Köppen*-Geiger: Cfa; Peel et al., 2007) with an average annual temperature varying between 18 and 20 °C and annual mean rainfall varying from 2000 mm to 2400 mm (PDM, 2009).

The study was approved by the Animal Research Ethics Committee (CEUA) under protocol number 112/2012. The minimum number of sera was calculated assuming 5% prevalence, 95% confidence interval, and 5% precision (Thrusfield, 2004). The dogs used in the VL survey were randomly sampled in rural and urban areas.

Blood samples from 252 dogs were collected by jugular venipuncture, and sera were separated and stored at −20 °C until use. Identification data from dogs (name, breed, sex, age, origin, clinical history, and presence of clinical signs) as well as the presence of forest, sylvatic or domestic animals, organic matter, water reservoirs, and sewage and garbage near the dwelling were recorded.

Three different approaches were used for VL diagnosis: enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and polymerase chain reaction (PCR).

Promastigotes of the reference strain *L. infantum* MHOM/FR/71/LRM75 grown at 24 °C in biphasic media brain–heart infusion broth (BHIB) were harvested from the exponential phase of growth and washed by centrifugation at 4 °C with sterile saline (0.9% and 0.3%) and PBS pH 7.2 and resuspended to a concentration of 10^7 promastigotes/mL. IFAT and ELISA antigen production processes were conducted as described by Castro et al. (2003) and Szargiki et al. (2009).

For ELISA, the sera samples were analyzed in 96-well flat-bottomed microtiter plates (high binding) sensitized with 500 ng/μL of protein diluted in coating buffer pH 9.6 and maintained overnight at 4 °C. After blocking with 2% casein in PBS, sera diluted 1:100 were added to each well and incubated at 37 °C for 1 h. The plates were then washed six times and incubated with rabbit peroxidase-conjugated 1:2500 anti-dog immunoglobulin G (IgG) antibody (Sigma). The reaction was revealed using hydrogen peroxide and ortho-phenylenediamine (OPD) and stopped with 20 μL of 2% sulfuric acid per well. Absorbance was determined in a spectrophotometer at 492 nm. A cutoff point for optimal sensitivity and specificity in ELISA tests was determined using receiver operating characteristic (ROC) curve analysis (Metz, 1978; Zweig and Campbell, 1993). The statistical analyses were performed using MedCalc Statistical Software (version 13.2.0).

For the IFAT assay, standardization of the technique was achieved with progressive dilutions of positive and negative control serum (1:20 to 1:320) against progressive dilutions of the conjugate (1:50 to 1:200), on five slides. The serum samples were screened in 1:40 dilution. The reactive sera were serially titrated up to a 1:640 dilution. The IgG conjugate (fluorescein isothiocyanate - Sigma) was used at 1:150 dilutions in 10% Evans Blue, from the previous titration. Each series of serum samples was run with positive and negative controls. The slides were analyzed using fluorescence microscopy (HBO 200 bulb and BG 12 filter).

DNA was extracted from the leukocyte layer of blood using the standard phenol–chloroform method (Sambrook and Russel, 2001). After ethanol precipitation, DNA was resuspended in 50 μL ultra-pure water, incubated with RNAse A (10 mg/mL) at 37 °C for 1 h, and stored at −20 °C until PCR realization. To amplify the 145 pb of the conserved region of the *L. infantum* DNA fragment, the primers RV1F and RV2R were used (Lachaud et al., 2002). The PCR was performed at a final volume of 25 μL containing 5 μL of DNA, 1× buffer, 0.2 mM dNTPs, 3 mM MgCl2, 0.16 pmol of each primer (F and R), 2 U Taq polymerase (Invitrogen), and water up to the final volume. The reaction condition consisted of an initial denaturation at 94 °C for 5 min. This was followed by 35 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 10 min. PCR product was analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under UV. *L. infantum* DNA was used as a positive control, and DNA from uninfected dogs, as well as the addition of no DNA, were used as negative controls of the assay. All DNA extraction procedures were performed in separate rooms to prevent contamination.

Prevalence was calculated according to the formula: number of positive dogs/sampled population. The Chi square test (χ^2) was used to compare prevalence rates among sex, age, breed, clinical signs, and place of origin. A p value of ≤ 0.05 was the criterion for significance.

Among the 252 dogs investigated, 74.7% were from rural areas with forests close to the home, the presence of other mammalians (opossums, rodents, horses and donkeys), and, in most cases, the presence of organic matter in the peridomiciliary area or close by. Concerning the canine population studied, 82.5% had no definite breed, 59.5% were male, 74.5% were aged 2 to 6 years old, and 86.5% were native to the region and had not been in another state for at least two years (Table 1). A statistically significant difference was observed, with a greater number of positive dogs among deer–hound breeds and those aged 7 to 10 years old ($p \leq 0.05$). This indicates that deer–hound breeds, known for hunting activity, are more susceptible to exposure to the vector because they live in woods and forests. More positive results among older dogs indicate a greater period at risk of being bitten and decreased immunity, which increases the chances of acquiring the disease.

For the ELISA method, the diagnostic performance was sensitivity of 100% (CI95%: 91.4–100%), specificity of 99.3% (CI95%: 97.4–99.9), area under the ROC curve (AUC): 0.999, Youden index: 0.993, $p < 0.0001$, likelihood ratio of a positive test (LR+): 135 and a negative (LR−): 0. Of the total serum samples surveyed, 41/252 were positive. Forty-three dogs were positive by IFAT assay, with 43 showing titers of 1/40, 33 with 1:80 and 25 ≥ 1:160. Fifty-five dogs were positive by PCR, 63.6% were asymptomatic (Table 2). Forty-eight dogs were positive for VL by both serological and molecular methods. The remaining 197 dogs were negative. Data analysis revealed that positive diagnosis was statistically associated with the symptomatology, breed, and age ($p \leq 0.05$) of the dogs.

Table 1
Epidemiological data and characteristics of the dogs studied.

Environmental conditions	Number (%) total residences	Number (%) positive dogs
Location of residence		
Rural area	56 (74.7)	35 (72.9)
Urban area	19 (25.3)	13 (27.1)
Presence of forest close to the residence		
Riparian forest	5 (6.7)	1 (2.1)
Native forest	37 (49.3)	25 (52.1)
Reforestation	15 (20.0)	12 (25.0)
Reforestation + native forest	14 (18.7)	10 (20.8)
No forests	4 (5.3)	0 (0.0)
Presence of forest within 300 m of the residence		
Presence	27 (36.0)	17 (35.4)
Absence	48 (64.0)	31 (64.6)
Presence of other animals		
Presence	71 (94.7)	48 (100.0)
Absence	4 (5.3)	0 (0.0)
Presence of organic matter in the yard		
Presence	73 (97.3)	48 (100.0)
Absence	2 (2.7)	0 (0.0)
Dog characteristics	Number (%)	
Breed		
No defined breed	208 (82.5)	35 (72.9)
Fila Brasileiro	17 (6.7)	0 (0.0)
Veadeiro Brasileiro	11 (4.4)	10 (20.8)
Others	16 (6.3)	3 (6.3)
Age		
Up to 1 year old	38 (15.1)	4 (8.3)
2 to 6 years old	188 (74.6)	23 (47.9)
7 to 10 years old	21 (8.3)	15 (31.3)
Over 10 years old	5 (2.0)	6 (12.5)
Sex		
Male	150 (59.5)	27 (56.3)
Female	102 (40.5)	21 (43.8)
Hunting dogs		
Yes	25 (9.9)	15 (31.2)
No	234 (90.1)	33 (68.8)

Among the 48 dogs positive by both serological and molecular methods, 19 (39.6%) presented clinical signs of VL. The most common clinical manifestations observed were skin alterations in 31.2% (alopecia, ulcerative lesions, and hyperkeratosis), weight loss (27.1%), onychogryphosis (25.0%), lymphadenopathy (20.8%), or anorexia (12.5%). Visceral leishmaniosis in dogs can exhibit a large spectrum of clinical presentations with variable degrees of severity (Dantas-Torres, 2006; Rondon et al., 2008; Baneth et al., 2008; Cunha et al., 2012).

Table 2
Number and percentage of dogs positive for visceral leishmaniosis following serological (IFAT and ELISA) and molecular (PCR) tests and relationship with clinical presentation.

Clinical form	Method	Number of positive dogs	% of positivity
Symptomatic	ELISA	19	46.4
	IFAT	17	39.6
	PCR	20	36.3
Asymptomatic	ELISA	22	53.6
	IFAT	26	60.4
	PCR	35	63.6

In our study, an average of 59.2% of positive dogs were asymptomatic (Table 2). These results were supported by other works (Dantas-Torres, 2006; Rondon et al., 2008; Cunha et al., 2012; Steindel et al., 2013), confirming that asymptomatic dogs act as parasite reservoirs, playing an important role in the maintenance of the parasite transmission cycle (Cardoso et al., 2012; Mohammadiha et al., 2013). The capacity to infect phlebotomines is higher for symptomatic (28%) than asymptomatic dogs (5.4%) (Michalsky et al., 2007). In a recent study in Florianopolis of 11 dogs with VL, 90.9%, including four asymptomatic dogs, presented high skin parasitism, as demonstrated by histopathology (Steindel et al., 2013). Therefore, under favorable conditions (higher sandfly density), asymptomatic dogs can contribute to parasite transmission, and the infection can spread and infect humans.

In summary, this work shows that of the 252 dogs investigated, 16.7% of them presented clinical signs of VL, 21% presented positive serology (ELISA + IFAT), and 21.8% were positive when tested by PCR. These results are supported by other studies in molecular epidemiology. For example, in Spain, 13% of dogs apparently presented clinical disease; 26% were seropositive and 63% were positive when PCR was used as a diagnostic technique (Solano-Gallego et al., 2001). In Greece, 12.3% of dogs presented anti-*Leishmania* antibodies and 63% were positive using the molecular technique (Leontides et al., 2002). This preliminary study confirms that the presence of CVL in the municipalities of São Miguel do Oeste and Descanso in the western region of Santa Catarina State means the occurrence of an outbreak is possible, as is the appearance of human cases in the future. The characteristics of the epidemiological cycle of VL in this region remain to be determined. A phylogeny study will provide important information regarding whether the parasite arrived in this region from neighboring Argentina or the States of Parana or Rio Grande do Sul.

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